**Anionic Detergent Mediated On-Slide Digestion: A Novel Approach to Collection of Archived Tissue for Bottom-Up Proteomics**

**Linan Wang**[1,2], Konstantin Shilo[3], Charles Hitchcock[3], Gerard Lozanski[3], Michael Alan Freitas[2]

[1] Biomedical Sciences Graduate Program, The Ohio State University. [2] Molecular Virology, Immunology & Medical Genetics, The Ohio State University. [3] Department of Pathology, The Ohio State University

**Abstract**

In the United States, cancer accounts for one out of every four deaths, and significant resources have been devoted to the improvement of diagnosis and treatment. Early and accurate diagnosis is critical in ensuring favorable outcome for the patient. Currently, the diagnosis and subtyping of numerous different cancers greatly rely on pathologist interpretation of patient biopsy samples. The importance of correctly subtyping a cancer is emphasized due to the differences in clinical outcome. This can be a challenging task since several of the diagnoses can have overlapping histological features. Through proteomic profiling of different cancer subtypes, biomarkers can be identified to facilitate diagnosis. This study describes a novel Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) method for characterizing archived formalin fixed paraffin embedded tissue (FFPE), accumulated over two decades.

Improved tissue staining to identify areas of interest on histological slides is a requirement for patient tissue use. Standard practice in pathology uses hematoxylin with eosin as a counter stain. However, this combination of stains is not feasible for proteomic studies due to eosin binding to proteins. Staining optimization was performed on reactive lymphatic tissue to test the ability to visualize the germinal center, mantle and interfollicular space. The studies show that toluidine blue is a suitable MS compatible counter stain in the visualization of lymph node structures. Mass

spectrometry experimentation demonstrates that protein identification and quantitation through spectral counting are not affected by the use of this combination of stains.

The second requirement for working with patient tissue is the ability to collect tissue from patient tissue slides for further sample processing. Biopsy samples are typically placed on positively charged glass slides. In cases where the tissue of interest is a minority in the total tissue on a slide, laser capture microdissection is used for specimen collection. Optimal LCM tissue capture requires special polyethylene naphalate (PEN) slides. Positive slides, used in standard pathology practice, are not amenable to laser capture and make tissue removal difficult. While mechanical collection of tissue with razor or scalpel is an option there are concerns of contamination due to the physical contact involved. A novel anionic detergent removal protocol was developed to neutralize the charge on the slide surface allowing for tissue release. An additional benefit to the use of anionic detergent is the ability to introduce endoproteases for protein digestion concurrent with tissue release. The final MS compatible sample can be collected by pipette from the slide surface for immediate MS analysis. Comparison of the collection of biopsy tissue using PEN slides, mechanical removal from positive slides and the novel on slide digestion/release approaches was conducted in this study. Patient tissue used for this study included normal lymph node, lung squamous cell carcinoma and thymoma subtypes A and B3. Our analysis showed consistent proteins coverage between the three methods. Quantitation by spectral counting revealed that protein abundances obtained from tissues processed by the mechanical and on slide digestion approaches were similar. Differences in protein abundance were noted, however, for the proteins obtained from PEN slices. The reason for this difference is hypothesized to be tissue association with the slide membrane.

The optimized approach was applied to proteomic studies of human lung neuroendocrine and thymoma cancers, which have not been previously studied by proteomics. Direct tissues studies are essential as there are no cell lines that mimic these tumors. The proteomic analysis showed that lung neuroendocrine and thymoma subtypes can be distinguished based on protein difference.

In summary, this methodology has enabled direct proteomic characterization of patient tissues in diseases where cell line models are lacking and diagnosis is histologically challenging and important to clinical outcome. These new methods will allow researchers to transition from *in vitro* cell line model to *in vivo* studies expanding our knowledge of the etiology of cancer while advancing clinical diagnosis and treatment.

**Introduction**

Currently in the United States, 14.5 million Americans have a history of cancer. This year it is expected that 1.6 million new cases will be diagnosed and over half a million people will die. Cancer is the second highest cause of mortality in the US behind heart disease and accounts for one in four deaths.[1] For many forms of cancer, the key to successful treatment and optimal outcome is early diagnosis and treatment. However, studies show that up to 60% of cancers are not diagnosed until after metastasis has occurred, greatly complicating therapy.[2] In the past decade great progress has been made in high throughput technologies for assessment of the DNA, RNA and proteins of whole cells in a single experiment, ushering in the –omics era. While the scope of this paper is to discuss proteomic research, it is important to understand that the –omics are all interlinked and combined play an important role in the regulation of homeostasis in a cell. Genomics enables the global study of DNA which serves as the blueprint templates for RNA transcripts. The RNA transcripts, studied globally by transcriptomics, in turn are translated into

proteins which serve as the effectors of many cellular functions. Proteomics research plays an important role in the clinical setting in two ways: biomarker discovery and therapeutic target discovery. Historically, genomics based biomarkers have been used in the diagnosis of disease through characterization of DNA shed by cells into the serum.[2] Assays search for DNA fragments carrying specific mutations. However, this method has an inherent flaw: while a disease associated DNA mutation may exist in a given individual, that specific mutation may not be transcribed or translated into proteins. Hence, detection of the mutated DNA does not indicate the disease state is present potentially leading to false positives in testing. Furthermore, DNA assays do not reflect protein post-translational modifications that regulate protein function.[3] Since proteins serve as the effectors of cellular function, a protein based approach demonstrating changes in protein structural abnormalities or quantity is a more accurate indicator of cellular dysfunction associated with disease. Also, a variety of drugs target proteins within a cell, as a means to either restore cellular homeostasis or activate cell death pathways to "cure" a disease.[4]

*Patient Tissues*

There are several advantages to the use of patient tissue over other model systems. Cell culture involving established cell lines has been a prominent source of research material over the years. However, there is debate on how well a cell line represents a human disease state.[5] Cell lines lack contact and signaling from neighboring cells, such as soluble factors, extracellular matrix molecules and cell-cell communications, which can lead to differences in gene expression from tissues.[6-8] Alternatively, xenografts involving the passage of human tumor cells into generations of mice has been used as a source of disease cells. However, this technique requires a large number of animals and is costly.[8]

Patient tissue is an attractive source of material for disease research for many reasons. First, human disease is heterogeneous. Molecular profiles from the cells of two patients with the same diagnosis are likely to show important differences. Thus, through use of samples from several different patients, researchers can account for this heterogeneity. While cell lines do not take into account the extracellular signaling that takes place in tissues, patient tissue are samples taken from their native environment including all of the associated factors such as cell-cell signaling, extracellular matrix and systemic signaling molecules transported by the blood stream. Several diseases currently do not have a corresponding cell line, therefore, patient tissue must be used. Finally, patient tissue can be archived and stored for decades. This allows for the collection of rare disease specimens over time. Also, patient tissue with longer history allows for longitudinal patient information to be collected detailing treatment outcome and prognosis.[9] Clinical patient data when combined with proteomic profiles is useful in the creation of retrospective studies.

Patient tissue is collected during biopsies and surgical resection. Unused tissue are catalogued and can be stored for some time prior to usage for research. Freezing and Formalin Fixation and Paraffin Embedding (FFPE) are two predominant methods for tissue preservation and storage. Although it has been documented by many researchers that frozen tissue produces the best sample quality downstream for DNA, mRNA and proteins, tissue freezing is costly in space and energy storage. The tissues must be kept at -80º C and can only be stored for one year.[10] Tissue freezing leads to loss of histological features, introducing challenges in the identification of areas of interest. Furthermore, collected patient tissue is typically not frozen unless experimental usage of the tissue is predetermined. The study described in this document uses FFPE tissue. FFPE storage involves the fixation of collected tissue in formalin (4% formaldehyde) then embedding in a paraffin block. FFPE tissue storage preserves tissue histology allowing for easy identification of

tissue collection boundaries.[11] The tissue blocks can then be sliced into thin slices and placed on positively charged SuperFrost Plus slides. Since patient tissue carries an inherent negative charge, the tissue is bound to the slide electrostatically without contaminating chemical adhesives.

*Bottom- Up Proteomics*

There are several techniques for the study of proteomics using mass spectrometry. Top down proteomics involves the use of intact proteins for mass spectrometry while middle down proteomics examines long peptides.[12] The mass spectrometry experiments described in this study involves the use of bottom up proteomics. In bottom up proteomics, proteins are digested into peptides and separated using liquid chromatography, then analyzed through tandem mass spectrometry. Tandem mass spectrometry collects peptide mass data (MS1) and peptide fragmentation data (MS2), in two back-to-back rounds of mass spectrometry. These data can then be used to obtain the peptide sequences leading to protein identification. Spectral counting is a label free quantitation method based on the principle that the number of spectra containing components of a given protein correlates with the abundance of that protein. The bottom up method is advantageous in the analysis of complex samples, such as the entire proteome of a cell, due to front end sample separation and protein quantitation.[13]

This manuscript outlines a process to take advantage of the wealth of patient samples collected by pathologists and preserved through FFPE techniques at several clinical institutions for disease research using bottom up proteomics. The techniques described will cover the visualization of tissue structures and the collection of tissue for the purposes of mass spectrometry proteomics.

**Methods**

*Patient Tissue*

Patient tissue used for this study is formalin fixed paraffin embedded. Tissues mounted on polyethylene napthalate (PEN) slides (Zeiss) were cut at 10 microns thickness. Tissue mounted on SuperFrost Plus slides were cut at 4 microns thickness. Staining studies were performed using reactive lymph node tissue mounted on PEN slides. Tissue collection studies were performed using thymoma subtypes A and B3, lung squamous cell carcinoma and lymph node tissue mounted on both PEN slides and Plus slides.

*Deparaffination and Staining*

Tissues were deparaffinized in three rinses of octane at 2 minutes each. Following deparaffinization, tissues were washed with decreasing gradients of ethanol: 100% (3 washes), 90% and 70% at 2 minutes each. Samples were then rinsed in water twice prior to staining (2 minutes). Stains were prepared in lock mailer tubes (Ted Pella Inc. 21096), slides are placed into stain for 5 seconds followed immediately by a 15 second water wash. Single staining was performed using hematoxylin (Vector H-3404) and toluidine blue (Fisher Scientific T161-25). This process was repeated for double staining. Staining process was completed through increasing ethanol gradients: 70%, 95% and 100% (2 washes) at 2 minutes apiece.

*Laser Capture Microdissection*

Tissue collection for the staining experiment was performed using laser capture microdissection. Reactive lymph node tissue germinal center, mantle and interfollicular space were collected on a PALM Microbeam IV, release 4.2 (Zeiss) and pressure catapulted into digestion buffer of 0.5% Rapigest (Waters Corporation) in 50 mM ammonium bicarbonate.

*Mechanical Tissue Collection*

Studies on tissue collection utilized mechanical tissue collection (macrodissection). In this study three cases of B3 thymoma was obtained from The Ohio State University Department of

Pathology for comparative study of tissue collection methods. Thymoma subtype A, lung squamous cell carcinoma and lymph node tissue were utilized to study generalized effects of tissue collection methods.

The tissue collection process is summarized in **Figure** 1. Tissues were cut at 10 micron thickness and mounted onto PEN slides and deparaffinized and stained using the process described in the previous section. A single PEN slide mounted specimen was used for each of the tissue samples. An incision surrounding the tissue of interest was made in the PEN membrane allowing the tissue to be lifted and collected for further sample processing. Tissue placed on SuperFrost Plus slides were cut at a thickness of 4 microns in triplicate for each tissue specimen. Following deparaffination and staining samples were collected by mechanical removal or on-slide digestion. For mechanical removal, tissue surrounding the area of interest was removed using a scalpel. The slide is then washed in ethanol and the tissue of interest is collected using dissection tools.

*PEN and Mechanical Collection Sample Digestion and Preparation*

Collected samples were dissolved in 0.5% Rapigest in 50 mM ammonium bicarbonate. Samples are boiled for 20 minutes then incubated for 2 hours at 60º C to reverse crosslinks from formalin fixation. For protein digestion 2 mg Trypsin (Promega) dissolved in 50 mM ammonium bicarbonate (pH ~7.4) was added to each sample. Samples were digested for 18 hours at 37º C. Rapigest and trypsin are inactivated through addition of acid lowering to pH ~2 and final peptide sample is dried and resuspended in 50 µL of HPLC water.

*On-Slide Digestion Sample Preparation*

On-slide digestion is a novel method described in this paper that combines the tissue collection and sample processing steps for maximum tissue collection with minimal outside contamination. Specimen tissues were cut to 4 microns thickness and mounted on Plus slides in

quadruplicate. Following deparaffination and removal of tissues of non-interest, slides were placed in lock mailer tubes (Ted Pella Inc.) and boiled for 20 minutes then incubated at 60º C for two hours. On-slide digestion involved the direct deposition of 20 µL digestion buffer (0.5 % Rapigest) with typsin (2 mg per sample). Slides were incubated at 37º C for 18 hours in the digestion process. Peptides are collected through rinsing the slide (rinses) and coverslip using rinses of 50 µL of 50 mM ammonium bicarbonate. Rapigest and trypsin were inactivated through addition of acid lowering the pH to ~2 and the final peptide sample was dried and resuspended in 50 microliters of HPLC water.

*HPLC and Mass Spectrometry*

Sample separation was performed using a Dionex 3000 UltiMate system using a C18 reverse phase column (Microm Bioresources Magic C18AQ, 200 µm x 150 mm, 200 Å). The mobile phases were water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The HPLC gradient consisted of an increasing percentage of the acetonitrile mobile phase over 5 hours. Mass spectrometry data collection was performed using a Thermo Scientific LTQ Orbitrap XL mass spectrometer. Data analysis for protein identification and spectral count analysis was performed through an in-house search engine, MassMatrix, with a Swiss-Prot human protein database[14-17].

*Detergent Removal of Tissue from Plus Slides*

The effectiveness of detergent ability to remove patient tissue from Superfrost Plus slides was assessed using 2 anionic detergents (Rapigest and sodium dodecyl sulfate) and 2 nonionic detergents (NP40 and Triton X). The detergents were placed on slides then incubated for 18 hours at 37º C. The slides were then rinsed with water and assessed for tissue removal.

**Results**

Experiments were performed to assess staining which allows for visualization of tissue histology that were compatible with mass spectrometry proteomics. Also, experiments compared the collection and sample preparation from PEN slides, mechanical tissue removal from SuperFrost Plus slides and a novel technique, on slide tissue digestion on SuperFrost Plus slides (**Figure 1**).

*Tissue Staining with Hematoxylin and Toluidine Blue*

Tissue staining results showed that hematoxylin staining alone is not sufficient to visualize the different structures found within the reactive lymph node (**Figure 2a and b**). While using diluted hematoxylin has improved the visualization of tissue structures the mantle regions remains difficult to visualize. Use of toluidine blue allows for visualization of the germinal centers (**Figure 2c**). When the tissue is first stained with hematoxylin then counter stained with toluidine blue all structures of the reactive lymph node can be discerned (**Figure 2d**).

Data analysis of mass spectrometry results demonstrate that there is a high degree of overlap in protein identification between the three compared staining methods: hematoxylin, toluidine blue and double staining using hematoxylin and toluidine blue (**Figure 3a**). Log- log plots of the spectral counts of the proteins identified comparing the toluidine blue single stain and the hematoxylin/ toluidine blue double stain shows a high degree of linear correlation (**Figure 3b**). Thus, the protein identification and spectral count quantitation of mass spectrometry data are not significantly affected by the different staining protocols tested.

*Tissue Collection using PEN and SuperFrost Plus Slides*

Successful tissue collection from the SuperFrost Plus slides was documented through a series of slide scans (Data not shown). Tissue collected from PEN slides was treated as complete collection, as incisions circumscribing the tissue of interest were made and tissue was directly

lifted off the slide supported by the PEN membrane. Correlation of the spectral count quantitation data between individual samples and grouped samples was performed (**Figure 4a**). Linear correlation of the log- log plots demonstrated that quantitation by spectral counting in mass spectrometry sample analysis was not adversely affected by the tissue collection process. Overlap between the three sample collection methods demonstrated that protein identification is independent of the method used (**Figure 4b**).

*Detergent Removal of Tissue from Plus Slides*

It was hypothesized that anionic detergents will interact with the positively charged SuperFrost Plus slides allowing tissue release from the slide. Experimentally, slides were incubated with anionic detergents (Rapigest and SDS) and nonionic detergents (NP40 and Triton X). This experiment demonstrated that the anionic nature of the digestion buffer used in studies was critical to the successful removal of tissue from the SuperFrost Plus slides (**Supplementary Figure 1**). Tissue was removed by washing when incubated with anionic detergents but not with nonionic detergents.

**Discussion**

Due to the heterogeneous nature of human tissue, histological visualization of tissue and structures are required to separate tumor cells from adjacent tissue. The pathology gold standard for visualization is the use of hematoxylin and eosin staining. Hematoxylin is a naturally occurring stain that is oxidized into hematein and mixed with metal ions that carry a positive charge. As a result, it stains negatively charged molecules within cells, such as nucleic acids, a purple blue color. Eosin is a synthetic stain targeting positively charged structures within cells such as lysine and arginine residues found in proteins. The binding of eosin to proteins leads to an alteration of mass, interfering with the mass spectrometry process. Since coverslips are not used on slides

destined for mass spectrometry proteomics, the issue is further complicated by darkening of stained tissue, leading to further loss of histological resolution. Historically, groups have used hematoxylin alone to stain tissues for mass spectrometry proteomics studies. However, in certain tissues the hematoxylin alone is insufficient in discerning the tissue structures, as demonstrated in reactive lymph nodes. Studies have shown that with a toluidine blue double stain lymph node structures are once again visible for sample collection. This suggests that combination staining using different stains, such as hematoxylin and toluidine blue, is a possible solution when working with difficult to visualize tissue structures.

Tissue collection methodology for bottom up mass spectrometry proteomics poses an interesting challenge. Laser capture microdissection (LCM) is a great advancement in the collection of small amounts of tissue using a laser system for both cold ablation and laser catapulting ensuring samples of great purity. However, during biopsy or surgical resection of larger tumors, the material placed on slides are predominantly tumor, making LCM unnecessary and cost ineffective. Thus, there exists a need to improve upon existing manual microdissection and macrodissection techniques. One existing practices is use of the Polyethylene Napthalate (PEN) slides in LCM for manual dissection if the tissue of interest is determined to be large enough. However, while existing collections of FFPE tissue can be readily placed on SuperFrost Plus slides, the PEN slides require manual effort by a pathologist leading to time consuming sample preparation. Additionally, this study has shown slightly lower data quality in both protein identification and spectral counts possibly due to PEN membrane sequestering of tissue from proteases or PEN polymer contamination of mass spectrometry samples. Alternatively, researchers have collected patient tissue from SuperFrost Plus slides through scraping the tissue off for further sample processing. Due to the sensitivity of mass spectrometry, keeping specimens contamination

free is essential. There exists concern around a dissecting instrument making physical contact with the tissue of interest. The proposed on-slide digestion has several advantages over existing methods. On-slide digestion can utilize patient tissue found on SuperFrost Plus slides, eliminating the PEN slide costs and pathologist workload. Also, on-slide digestion insures the digestion of the entire tissue sample while on the slide without mechanical contact from a dissection instrument, reducing the risk of contamination. In summary, on-slide digestion is a viable method for performing bottom up proteomic experiments using archived FFPE tissues.

The power of this method was demonstrated in a biomarker discovery experiment involving thymoma subtyping. While the thymoma is neither the most common nor the highest source of cancer mortality it does not have a representative cell line, thus, it has never been characterized molecularly. Thymomas are divided into five subtypes with varying clinical outcomes ranging from 100% survival rate for thymoma type A to 40% survival rate for thymoma type B3. Current subtype diagnosis involves pathologist histological interpretation of diseased tissue. However, the overlapping histological features between the different subtypes make objective diagnosis challenging. Thus, there exists a need for a molecular assay for a definitively objective diagnosis. In this experiment, we tested the five different thymoma subtypes with six patient cases apiece using the methods described in this manuscript. Proteins were identified and quantified then sorted through cluster analysis of the different patient samples. Analysis shows grouping of the different subtypes, suggesting that proteomic assays for distinguishing different subtypes is a possibility (**Figure 5**). Also, several proteins which are known biomarkers or cancer therapeutic targets found in tumors of other parts of the body were successfully detected in our study as potential markers for thymoma subtyping. This study further demonstrates the value of

the methods proposed in this manuscript for use with proteomics research in biomarker and therapeutic target discovery.

## Conclusion

The staining and tissue collection methods described in this manuscript address the challenge faced by researchers attempting to use archived FFPE tissue. These advancements will open the doors for proteomics research on FFPE tissue from previously uncharacterized diseases where cell lines are not readily available.

## Acknowledgements

The project described was supported by Award Number Grant TL1TR001069 from the National Center For Advancing Translational Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Advancing Translational Sciences or the National Institutes of Health.

**Figure 1: Tissue Collection Methods Experimental Schematic**
A. Collection of tissue from PEN slides. B. Mechanical collection of patient tissue from SuperFrost Plus slides using a dissecting instrument. C. On slide digestion of patient tissue.

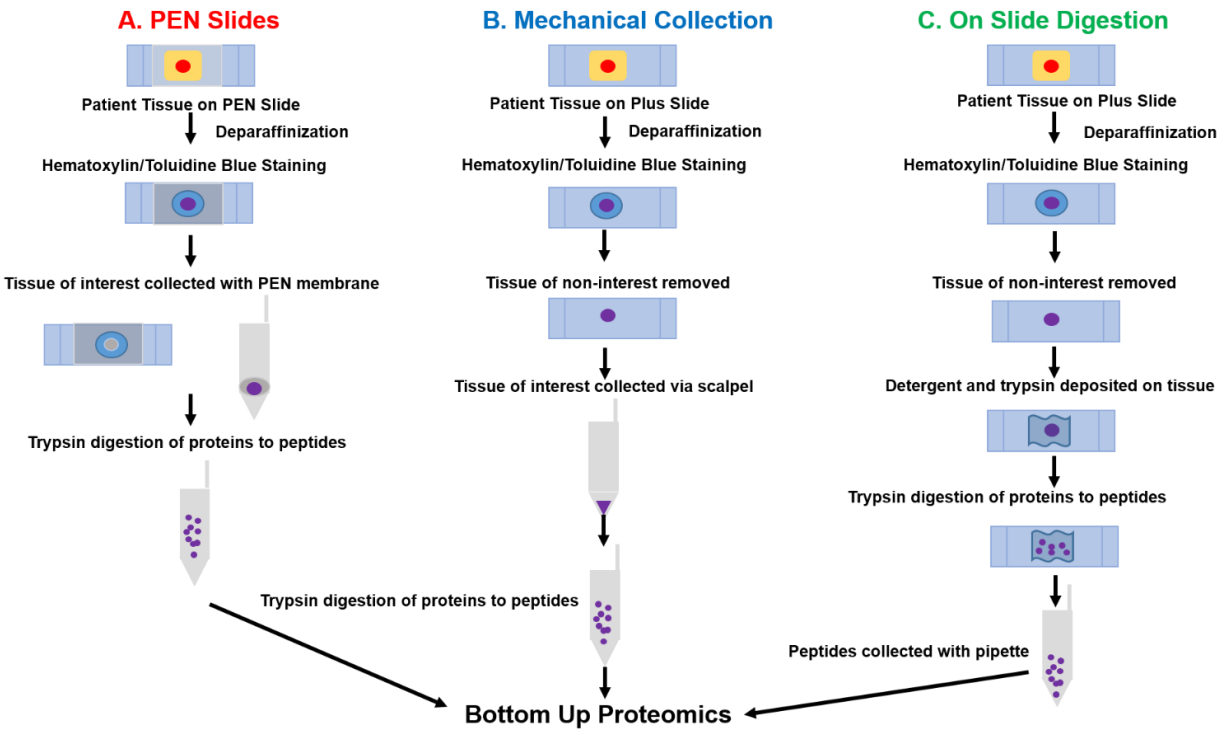


**Figure 2: Tissue Staining of Reactive Lymph Nodes**
A. Hematoxylin and eosin stain of reactive lymph node tissue. H&E stains are the gold standard for staining in histology. B. Hematoxylin single stain. Note the lack of visibility of the tissue structures. C. Toluidine blue single stain. While the germinal centers can be visualized, the mantle region remains unclear. D. Hematoxylin and toluidine blue double stain. The germinal center, mantle and interfollicular space are visible using this staining method.

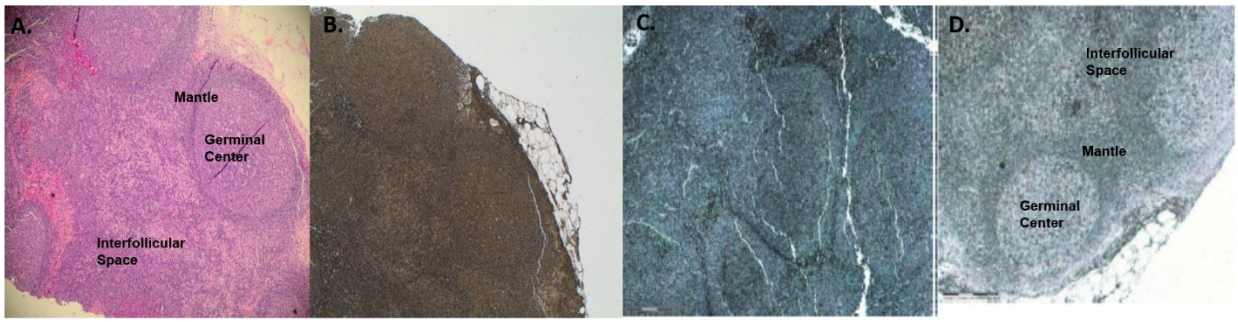


**Figure 3: Protein Identification and Spectral Count Quantitation in Reactive Lymph Nodes are Independent of Staining Method**

A. Venn diagram comparing the proteins identified between the three different staining methods: hematoxylin single stain, toluidine blue single stain and hematoxylin and toluidine blue double stain shows large overlap, indicating that the proteins detected are consistent in each of the sample staining methods. B. The log-log plots of the spectral counts compare the quantitation of proteins between the samples. The linear relationship indicates that the spectral counts between samples are comparable.

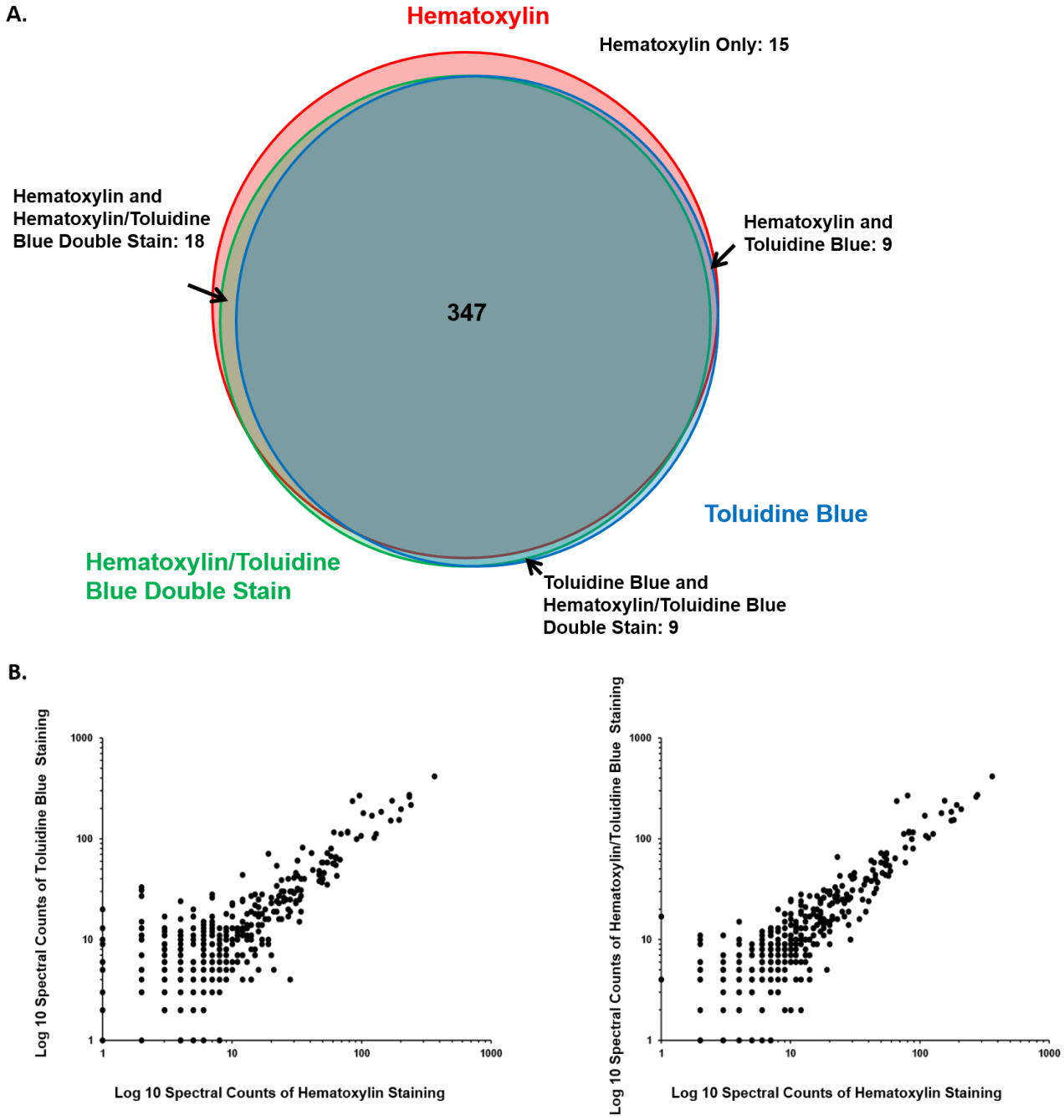


**Figure 4: Protein Identification and Spectral Count Quantitation in Reactive Lymph Nodes are Independent of Tissue Collection Method**

A. The log-log plots of the spectral counts compare the quantitation of proteins between the samples. Top: Spectral count comparisons between the three different tissue collection methods using cumulative data from three thymoma B3 specimens. Bottom: Spectral count comparisons between the collection methods using cumulative data from thymoma B3 and A, lung squamous cell carcinoma and lymph node tissue. The linear relationship indicates that the spectral counts between samples are comparable. B. Venn diagram comparing the proteins identified between the three different tissue collection methods: PEN slide, mechanical collection and on-slide digestion shows large overlap, indicating that the proteins detected are consistent across each of the sample collection methods.

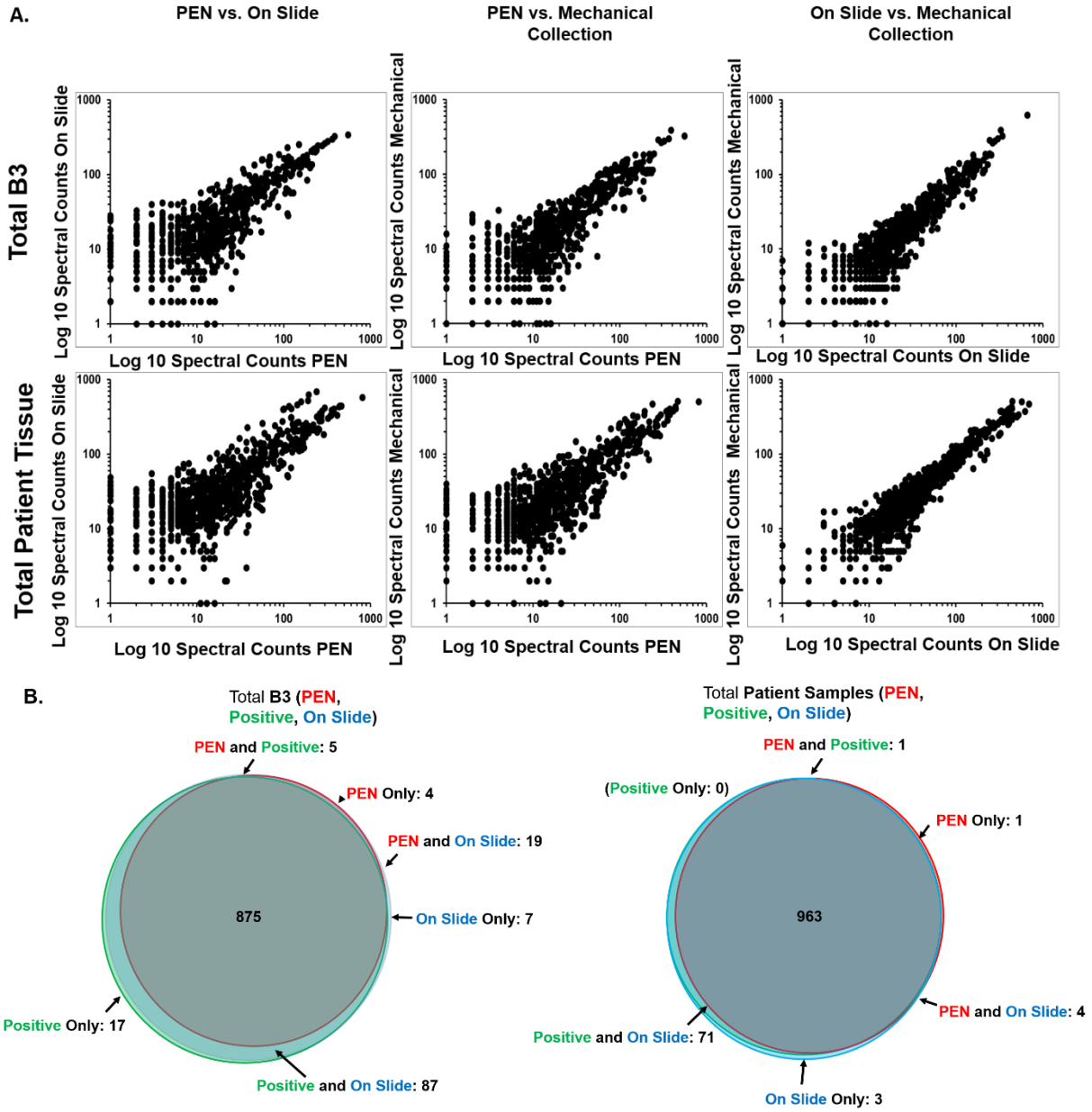
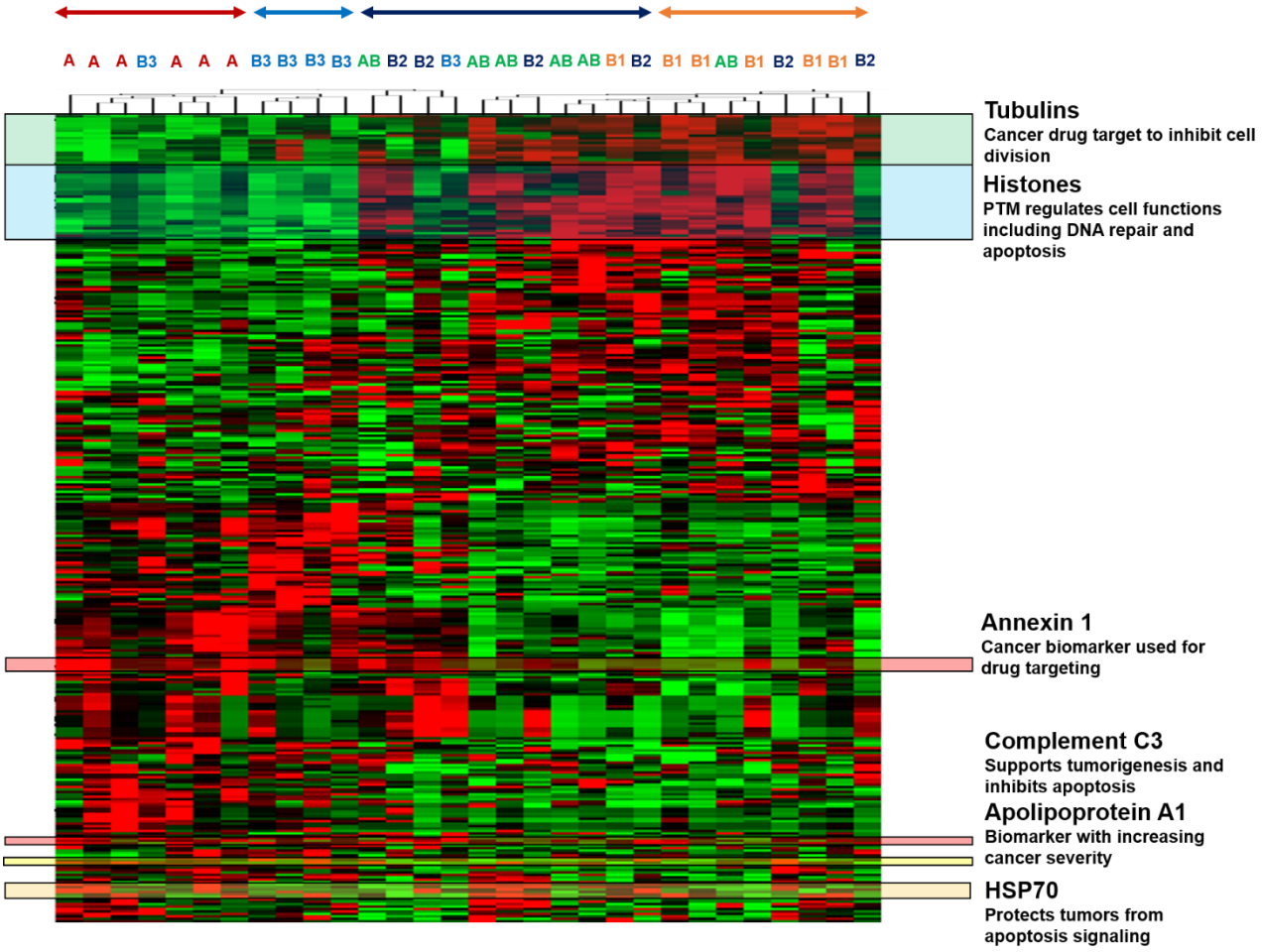


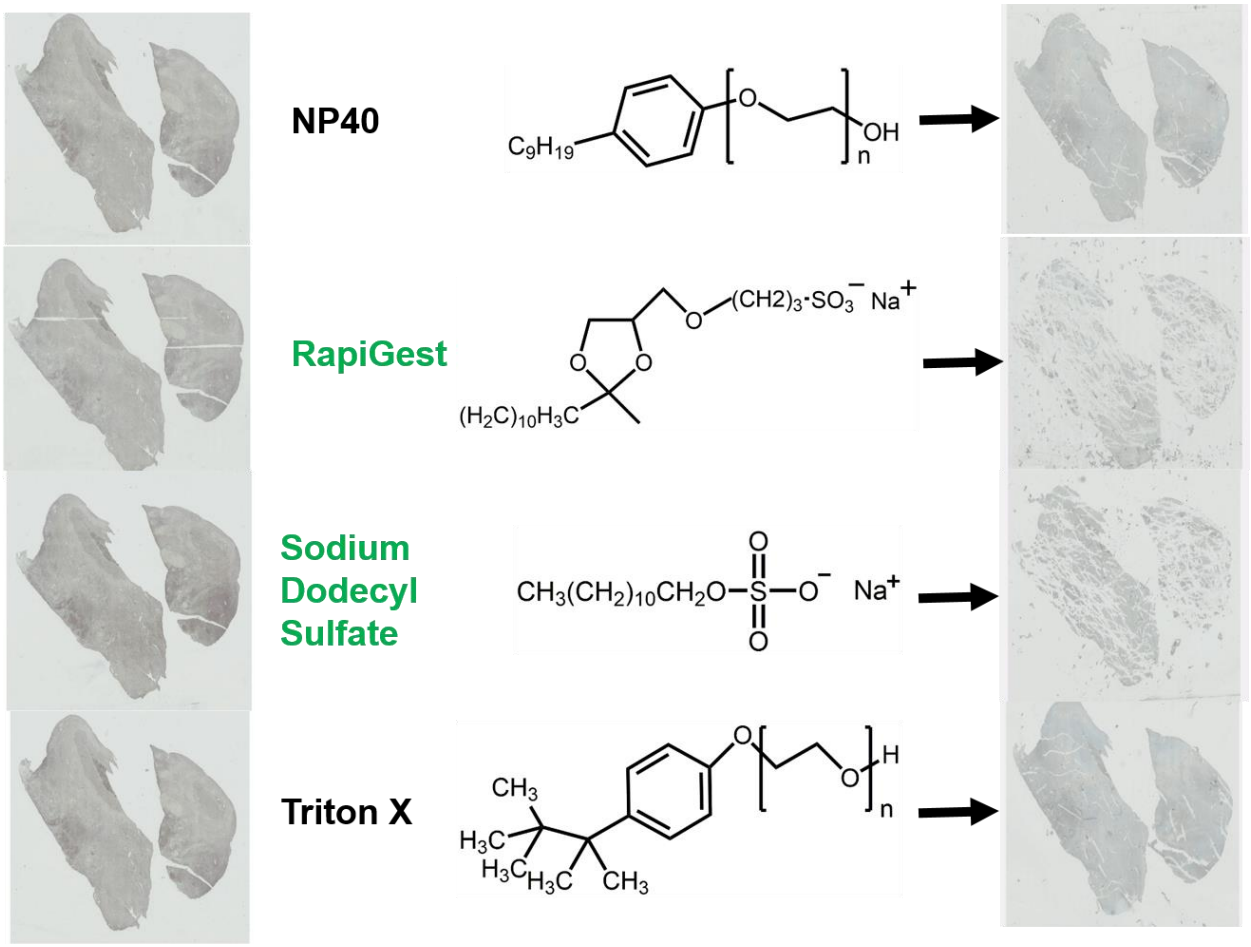
**Figure 5: Cluster Analysis of Thymoma Subtype**

6 patient specimens for each of the thymoma subtypes were analyzed using the method described in this manuscript. Cluster analysis grouping showed the B3 specimens clustering together, suggesting that they can be distinguished proteomically. Proteins known to have biomarker or therapeutic target utility in other cancers were identified as differentially abundant across the thymoma samples, showing difference between the subtypes.



**Supplementary Figure 1: Tissue Collection from SuperFrost Plus Slides is Mediated by Anionic Detergents**

Incubation of patient tissue in anionic detergents (Rapigest and sodium dodecyl sulfate) and nonionic detergents (Triton X and NP40) followed by washes show that the tissue release from Plus slides only occurs in the presence of anionic detergents. This supports the hypothesis that the anionic detergents binds to the positively charged SuperFrost Plus slides mediating tissue release for collection.



**References**


1 Cancer Facts and Figures 2015. *American Cancer Society* (2014).

2 Wulfkuhle, J. D., Liotta, L. A. & Petricoin, E. F. Proteomic applications for the early detection of cancer. *Nature Reviews Cancer* **3**, 267-275, doi:10.1038/ncr1043 (2003).

3 Gutstein, H. B. & Morris, J. S. Laser capture sampling and analytical issues in proteomics. *Expert review of proteomics* **4**, 627-637, doi:10.1586/14789450.4.5.627 (2007).

4 Liu, N. Q. *et al.* Proteomics pipeline for biomarker discovery of laser capture microdissected breast cancer tissue. *Journal of mammary gland biology and neoplasia* **17**, 155-164, doi:10.1007/s10911-012-9252-6 (2012).

5 Craven, R. A. & Banks, R. E. Laser capture microdissection and proteomics: possibilities and limitation. *Proteomics* **1**, 1200-1204, doi:10.1002/1615-9861(200110)1:10<1200::AID-PROT1200>3.0.CO;2-Q (2001).

6 Simone, N. L., Bonner, R. F., Gillespie, J. W., Emmert-Buck, M. R. & Liotta, L. A. Laser-capture microdissection: opening the microscopic frontier to molecular analysis. *Trends in genetics : TIG* **14**, 272-276 (1998).

7 Fend, F. & Raffeld, M. Laser capture microdissection in pathology. *Journal of clinical pathology* **53**, 666-672 (2000).

8 Curran, S., McKay, J. A., McLeod, H. L. & Murray, G. I. Laser capture microscopy. *Molecular pathology : MP* **53**, 64-68 (2000).

9 Hunt, J. L. & Finkelstein, S. D. Microdissection techniques for molecular testing in surgical pathology. *Archives of pathology & laboratory medicine* **128**, 1372-1378, doi:10.1043/1543-2165(2004)128<1372:MTFMTI>2.0.CO;2 (2004).

10 Caldwell, R. L. & Caprioli, R. M. Tissue profiling by mass spectrometry: a review of methodology and applications. *Molecular & cellular proteomics : MCP* **4**, 394-401, doi:10.1074/mcp.R500006-MCP200 (2005).

11 Gillespie, J. W. *et al.* Molecular profiling of cancer. *Toxicologic pathology* **32 Suppl 1**, 67-71 (2004).

12 Han, X., Aslanian, A. & Yates, J. R., 3rd. Mass spectrometry for proteomics. *Current opinion in chemical biology* **12**, 483-490, doi:10.1016/j.cbpa.2008.07.024 (2008).

13 Gregorich, Z. R., Chang, Y. H. & Ge, Y. Proteomics in heart failure: top-down or bottom-up? *Pflugers Archiv : European journal of physiology* **466**, 1199-1209, doi:10.1007/s00424-014-1471-9 (2014).

14 Xu, H., Zhang, L. & Freitas, M. A. Identification and characterization of disulfide bonds in proteins and peptides from tandem MS data by use of the MassMatrix MS/MS search engine. *Journal of proteome research* **7**, 138-144, doi:10.1021/pr070363z (2008).

15 Xu, H., Yang, L. & Freitas, M. A. A robust linear regression based algorithm for automated evaluation of peptide identifications from shotgun proteomics by use of reversed-phase liquid chromatography retention time. *BMC bioinformatics* **9**, 347, doi:10.1186/1471-2105-9-347 (2008).

16 Xu, H. & Freitas, M. A. A mass accuracy sensitive probability based scoring algorithm for database searching of tandem mass spectrometry data. *BMC bioinformatics* **8**, 133, doi:10.1186/1471-2105-8-133 (2007).

17 Xu, H. & Freitas, M. A. Monte carlo simulation-based algorithms for analysis of shotgun proteomic data. *Journal of proteome research* **7**, 2605-2615, doi:10.1021/pr800002u (2008).